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## **SPECTROSCOPIC TRENDS FOR THE DETERMINATION OF ILLICIT DRUGS IN ORAL FLUID**

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### **ABSTRACT**

The present work aims to review all the papers published so far, focused on the determination of drugs of abuse in oral fluid. This fluid provides a simpler, faster and more controllable sampling in comparison with the other biological fluids such as blood or urine. Actually, the main goal of the researchers is to lower the limit of detection (LOD) to detect quantities of drugs smaller than the cut-off limits established by law for drug controls. Advances in Raman, Infrared (IR) and Nuclear Magnetic Resonance (NMR) spectroscopy applications are here discussed. Surface-enhanced Raman spectroscopy (SERS) has been shown as the most sensitive technique for the detection of illicit drugs in oral fluid. The use of IR spectroscopy for determining drugs of abuse in oral fluid is growing although the LODs obtained until now do not yet satisfy the necessities in the forensic field. Finally, NMR spectroscopy has been seldom used to determine drugs in oral fluid. Another future trend seems to be related with the use of portable instrumentation, which would permit to perform in situ analysis. This last application seems to be particularly promising to perform roadside drug tests and to identify overdose drugs in patients in emergency conditions.

## **Introduction**

The determination of illicit drugs in oral fluid is a topic of growing interest, above all in forensic and clinical fields where the non-destructive character of spectroscopic techniques is very important. This characteristic allows to carry out different analyses, even if the quantity of sample available is very small. It should be emphasized that, throughout the text, with “non-destructive” we mean that spectroscopic techniques permit to reanalyze the sample for further studies. Instead, in other techniques like Gas Chromatography coupled with Mass Spectrometry (GC-MS) or Liquid Chromatography coupled with Mass Spectrometry (LC-MS) the sample analyzed is destroyed. This not exclude the fact that, before the analysis, the sample may need a pretreatment which modifies its original nature, as it happens with oral fluid samples in the works here reviewed.

Until now, several approaches for detecting drugs of abuse and medicinal drugs in oral fluid have been developed. The majority of these are based on immunological procedures or chromatographic techniques coupled with mass spectrometry or tandem mass spectrometry. Enzyme immunoassays (EIA) are based on the competition for binding to an antibody between an enzyme-labelled antigen and an unlabeled drug, in proportion to their concentration. The interaction is so detected using an antibody-coupled enzyme which converts a colorless substrate to a colored or luminescent product [1-3]. The problem of these assays is that there is a great probability to obtain false negative or false positive results, so EIA are used as preliminary screening approaches, which are then followed by a chromatographic technique to confirm the results. Beside the well-known GC-MS methods, also some LC-MS methods have been described. In contrast to the immunoassays, these chromatographic techniques require sample pretreatment that makes the analysis time consuming. Since all these methods have been recently and extensively reviewed [4-9], the aim of this work is to review the spectroscopic approaches developed so far to determine drugs in oral fluid.

Up to date, Ultraviolet-Visible (UV-VIS) and fluorescence spectroscopy have mainly been used as detectors for immunoassays or separation techniques. These studies are collected in recently published reviews dealing on the determination of cocaine and its metabolites [7] or opiates and opioids [8] in biological samples. For this reason, we will focus our attention on Raman, IR and Nuclear Magnetic Resonance (NMR) applications dealing on the detection of illicit drugs in oral fluid. A description of the current challenges in the determination of drugs of abuse in oral fluid is firstly provided. However, the principal objective of this review is to summarize the state of art of this topic, providing a critical opinion about the validity and applicability of the studies reported up to date.

## **Current challenges in the determination of illicit drugs in oral fluid**

In the last years, oral fluid has become an important alternative to blood and urine as a matrix for therapeutic drug screening, work-place monitoring and drug of abuse testing. Although in the reviewed papers, the terms “oral fluid” and “saliva” are often used indifferently, in this work we have selected the term “oral fluid”. “Saliva” means specifically the secretion of the salivary glands (parotids, submandibular and sublingual), whereas the oral fluid also contains mucins (high molecular weight proteins), mucosal transudate and crevicular fluid (also called gingival fluid, which serves as a defence mechanism against infections, by carrying antibodies and other substances between the connective tissue and sulcus or pocket). The oral fluid is a highly aqueous liquid, natural ultra-filtrate of plasma, and offers different advantages: it is relatively

clean because is colorless and it can be collected in a simple, inexpensive and non-invasive manner by nonmedical personnel. Oral fluid sampling can be closely supervised without invasion of privacy to prevent substitution, adulteration or dilution of the sample, which could happen in urine analysis, and to avoid the risk of infection, possible during a drawing blood. The principal drawback of the use of oral fluid is the small quantity of sample collectable. This problem could be even enhanced by diseases or some common habits like smoking, using mouthwashes or medicinal drugs which may reduce salivation. Some disadvantages are also correlated to oral contamination from certain routes of administration (smoking, snorting or oral ingestion) and to the sampling method that may cause variations in salivary flow and pH [4, 10-13]. Finally, drug concentrations in oral fluid are different than the ones in traditional biofluids. As example, for basic drugs, concentrations in oral fluid are usually higher than in blood and these compounds could be easily detected even from a small sample volume. For acidic drugs, instead, concentrations in oral fluid can be much lower [14]. Consequently, the analytical methodology has to be highly sensitive to be able to detect and quantify multiple compounds simultaneously from a single sample [15]. However, differently from the urine in which detection times of drugs are between 2 days and 3 months, drug times of detection in oral fluids (2-48 hours) are similar to those in blood (24-48 hours) and so it could be used for the analysis when an indication of recent drug use is required [16].

Common methods for oral fluid collection are spitting, draining, suction and wiping the oral cavity with a swab. Usually spitting itself is a sufficient stimulus to elicit a flow, but sometimes the sample volume is insufficient for the analysis, so the flow can be stimulated mechanically (e.g. chewing a gum or an inert material) or chemically (e.g. placing a sour candy or citric acid crystals in the mouth). However, the sample obtained differs in composition from oral fluid collected without stimulation. In fact, the stimulation modifies the pH gradient between this fluid and the plasma, so the diffusion and consequently the concentration of the drug are reduced [5, 13].

Commercial swabs consist of a sorbent material that becomes saturated in the mouth of the donor and then removed from the oral cavity. The fluid is later recovered by centrifugation or by applying a pressure, but significant differences in the recovery percentage from the sorbent material for various drugs have been reported [5]. In order to study the performances of these different collection approaches, different common substances are under investigation: amphetamines, benzodiazepines, cannabinoids, cocaine, opiates, barbiturates, sedatives, anesthetics, other psychoactive drugs like hypnotics, hallucinogens, antidepressants or antipsychotics and all their metabolites [5, 13].

The conventional mechanism of drug transport from the blood into the oral fluid is the passive diffusion across a concentration gradient. It is regulated by physicochemical properties such as molecular weight, dissociation constants, lipid solubility and protein bonds of the substances, and by other factors like the oral fluid flow rate or the pH values in blood (~7.4) and oral fluid (~6), that depend on the manner in which the sample has been collected [10]. Generally, the oral fluid contains the parent drug because of its higher lipophilicity and, consequently, its higher potential for passive diffusion. In fact, it has been demonstrated that after the intake of a drug by smoking, nasal insufflation or oral ingestion, a contamination of the oral cavity can lead to dramatically elevated parent drug concentrations in oral fluid leading to a misinterpretation. Since this happen only shortly after the abuse, the sample should be collected within about 2 hours from the administration. There is the evidence that when a given drug is detected in oral

fluid, there is a high probability that the subject is under the pharmacologic effect of that drug [10, 17-18].

### Spectroscopic approaches to detect drugs of abuse in oral fluid

Below, a comprehensive summary of the studies applying spectroscopy to the analysis of drugs of abuse in oral fluid has been made. The works reviewed have been subdivided according to the technique utilized. In turn, the techniques have been listed according to their sensitivity.

Raman spectroscopy in SERS working mode permitted to obtain the lowest LODs, followed by IR and NMR spectroscopy. Table 1 and Table 2 resume these information showing the studied drug, the LOD achieved and the cut-off limits established by the Substance Abuse and Mental Health Service Administration (SAMHSA). Table 1 also collects the SERS nanoparticles type and lasers wavelength used. Table 2 also shows the mode of IR spectroscopy employed, the lasers wavelength used and the range in which the analyses have been conducted.

As visible, most of the papers here reported are focused on the detection and quantification of cocaine in particular, since the highest percentage of drug overdoses is due to its intake. Actually, cocaine is the second most trafficked drug in the world after the marijuana.

**Table 1.** Illicit drugs studied by SERS and measurement conditions

Illicit drug	SERS NPs	Laser	LOD	Cut-off*	Reference
Cocaine	Gold- and Silver-doped sol-gel	785 nm	250 µg/mL	8 ng/mL	23
Cocaine	Gold- and Silver-doped sol-gel	785 nm	50 ng/mL	8 ng/mL	24
Cocaine	Gold-doped sol-gel	785 nm	50 ng/mL	8 ng/mL	25
Diazepam	Gold-doped sol-gel	785 nm	100 ng/mL	20 ng/mL	26
Methamphetamine	Silver in suspension	633 nm	1.5 ng/mL	15 ng/mL	27
Cotinine	Fe <sub>3</sub> O <sub>4</sub> dotted with Gold	785 nm	8.8 ng/mL	18 ng/mL	28
Benzoyllecgonine	Fe <sub>3</sub> O <sub>4</sub> dotted with Gold	785 nm	29 ng/mL	8 ng/mL	28

\*Established by Substance Abuse and Mental Health Services Administration (SAMHSA).

**Table 2.** Illicit drugs studied by infrared (IR) spectroscopy and measurement conditions

Illicit drug	Technique	Laser	Range	LOD	Cut-off*	Reference
Cocaine	ATR-FTIR spectroscopy	----	2300-900 $\text{cm}^{-1}$	20 $\mu\text{g/mL}$	8 ng/mL	30
Cocaine	ATR-FTIR spectroscopy	----	2300-900 $\text{cm}^{-1}$	< 10 $\mu\text{g/mL}$	8 ng/mL	31
Cocaine	ATR-FTIR spectroscopy	1750 $\text{cm}^{-1}$	----	3 $\mu\text{g/mL}$	8 ng/mL	32
Cocaine	Transmission Cell FTIR spectroscopy	1750 $\text{cm}^{-1}$	----	< 10 $\mu\text{g/mL}$	8 ng/mL	32
Cocaine	On-chip IR spectroscopy	1750 $\text{cm}^{-1}$	2000-900 $\text{cm}^{-1}$	500 $\mu\text{g/mL}$	8 ng/mL	33
Cocaine	ATR-FTIR spectroscopy	1750 $\text{cm}^{-1}$	1850-1000 $\text{cm}^{-1}$	370 ng/mL	8 ng/mL	35
Cocaine	QCL-based MIR spectroscopy	1720 $\text{cm}^{-1}$	----	100 $\mu\text{g/mL}^{**}$	8 ng/mL	36
MDMA	IMS direct analysis	----	----	160 ng/mL <sup>**</sup>	15 ng/mL	37
MDMA	IMS after LLME	----	----	11 ng/mL <sup>**</sup>	15 ng/mL	37
MDMA	ATR-FTIR spectroscopy after LLME	----	4000-650 $\text{cm}^{-1}$	320 ng/mL <sup>**</sup>	15 ng/mL	37

\*Established by Substance Abuse and Mental Health Services Administration (SAMHSA).

\*\*Estimated by interpolation into the calibration curves reported in [36] and [37].

### ***Raman spectroscopy***

Raman spectroscopy is a type of vibrational spectroscopy based on the inelastic scattering of laser light after its interaction with the molecules. Raman scattering (inelastic) occurs when incident photons with wavenumber  $\bar{\nu}$  interact with the molecules yielding scattered photons with shifted wavenumbers ( $\bar{\nu} \pm \Delta\bar{\nu}$ ), and differs from Rayleigh scattering (elastic) in which the incident and scattered photons have the same frequency. The Raman shift ( $\Delta\bar{\nu}$ ) represents the energy of the molecular vibrations within the sample, while Raman spectra provide information regarding the molecule. The frequencies of the molecular vibrations depend on the atom masses, on their geometric arrangement and on the strength of their chemical bonds. So, with Raman spectroscopy, it is possible to know the structure and conformation of the molecule; the interaction between molecules; the surrounding environment; and the physical state and condition of the matter [19]. The method provides a unique spectrum for each compound and is highly sensitive to slight differences in chemical composition and crystallographic structure. This is very useful for the investigation of illegal drugs because even small discrepancies allow one to obtain information regarding the synthesis method of the drug [20].

This technique offers a non-destructive and rapid method for substances identification and requires only small quantities of sample, which almost always does not need a pretreatment, so small traces of evidence could be exploited for different analyses.

Raman Spectroscopy can analyze liquids, tablets and powders, and its signal is not much affected by those of plastic and glass containers. Then, the analysis can be achieved without removing the sample from its packaging, avoiding contamination and ensuring the integrity of the sample. However, the main disadvantages of Raman spectroscopy are the possibility of having fluorescence interference and the weakness of the Raman Effect in absence of resonance and/or surface enhancement. Fluorescence contributions are commonly minimized using numerical methods to eliminate fluorescent background from previously recorded spectra. Instead, since the probability of observing the Raman Effect is approximately  $10^{-6}$ - $10^{-9}$  per incident photon, the weakness of the signal imposes the use of a highly sensitive light detector or the application of a particular working mode of Raman spectroscopy called SERS [21].

In this last case, the enhancement comes from the electromagnetic interaction of light with metals (usually gold and/or silver nanoparticles), which produces large amplifications of the laser field through excitations generally known as “plasmon resonances”. To profit from these, the sample must typically be adsorbed on the metal surface, or at least be very close to it (typically  $\sim 10$  nm maximum). The enhancement factor (EF) is defined as the ratio of SERS signal to the Raman signal obtained for the same molecule in the absence of the SERS substrate, with all other conditions being identical. Finally, SERS signal can achieve scattering upward 8 orders of magnitude greater than the traditional Raman spectroscopy [22].

Nevertheless, until now, only few published studies deal specifically on the detection of drugs in oral fluids by Raman spectroscopy (see table 1) and all have been performed with the SERS mode [23-28].

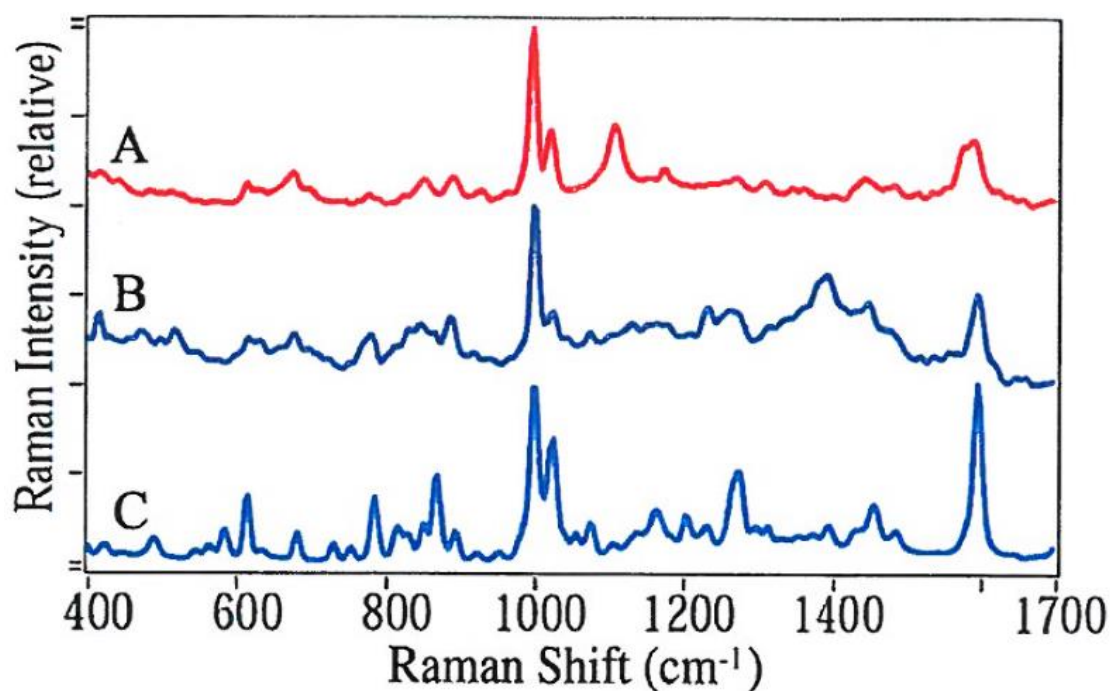
The first study about the determination of drugs and metabolites in oral fluid by SERS was reported by Shende et al. [23]. They investigated metal-doped sol-gels to separate drugs and their metabolites from oral fluid and to generate SERS spectra. The sol-gel was incorporated in a chip, where no more than a drop of sample was required. Initially they developed a SERS-active medium composed of silver particles incorporated on a porous glass structure. With this device several barbiturates (secobarbital, amobarbital and phenobarbital) were successfully identified

using a 785 nm diode laser. However, the spectra obtained were very similar among themselves and a chemical separation was required in order to differentiate the parent drug from its metabolites. Towards this aim, the SERS-active sol-gels were incorporated into capillaries and used to simultaneously separate and determine the different substances. Nevertheless, this approach had two limitations: the analytes had to be active on the same metal-doped sol-gel and the amount of analyte injected into the capillary had to be precisely controlled. To resolve these problems, they incorporated in series multiple segments (plugs) of SERS-active sol-gels with different chemical selectivity (gold and silver) in a capillary. The sol-gels were designed to extract and concentrate the target analyte, increasing the sensitivity.

Furthermore, as a practical matter, if the sol-gel segments were small, the sol-gel did not need to be scanned for the target analyte, but they were only measured at the plugs and this also eliminated the need of precise flow control. To test this developed device, three mixtures 50/50 (v/v) of cocaine/cafeine, caffeine/phenobarbital and phenobarbital/cocaine were prepared. It was noted that caffeine produced SERS spectra on the gold-doped segment while cocaine and phenobarbital were active on the silver-doped-segment. However, differently from the two first mixtures, the separation and detection of cocaine and phenobarbital was not successful. The device initially designed was then modified putting the SERS-active segments in parallel instead of in series. Another sol-gel segment was also added to first separate the substances, before their introduction in the extraction segments. This new design allowed to separate and measure cocaine and phenobarbital and the entire analysis took less than 5 minutes. These measurements were hence repeated spiking an aliquot of oral fluid with the same three substances before mentioned to produce a sample that was 0.25 mg/mL for each drug. Again, caffeine and phenobarbital were immediately determined while cocaine gave problems. Finally, the use of sulfuric or acetic acid combined with heat or sonication, before the injection in the capillary, made possible to separate cocaine from the mucins of oral fluid and to detect successfully concentrations in the order of  $\mu\text{g/mL}$  (see table 1) [23].

Subsequently, an attempt to produce a SERS-active sampling system with the aim of developing a roadside drug-screening device was made by Inscore et al. [24]. They detected cocaine in oral fluid and then applied the same method also to the detection of other illicit drugs such as amphetamine, methadone, phencyclidine and diazepam (which really is a therapeutic drug but, due to its effects, is commonly used in an illegal way). In this study, the enhancement of the signal was provided by electronegative gold and electropositive silver nanoparticles used to attract charged chemical groups and to ensure the interaction of the drugs with the plasmon field. The nanoparticles were incorporated in a porous glass structure immobilized in a glass capillary and the sample was then injected in and immediately measured without the necessity to be dried. Although many drugs were highly active on silver, the majority of them were active on gold. Figure 1 shows the SERS spectra of cocaine on gold- and silver-doped sol-gel and the conventional Raman spectrum acquired with a Fourier Transform Raman spectrometer with a 785 nm laser excitation. EFs were calculated to be  $3.13 \cdot 10^6$  and  $1.13 \cdot 10^4$ , respectively, by comparing SERS spectra to the normal Raman spectrum. The calculation was made taking into account the laser power, the concentrations, and the relative intensities of the  $999\text{ cm}^{-1}$  peak. In fact, since this peak is the most characteristic of cocaine, spectra were normalized for the plot by setting the baseline to 0 and the  $999\text{ cm}^{-1}$  peak height to 1. Due to the better EF, only gold-doped sol-gel capillaries were chosen for the further method development.





**Figure 1.** Comparison of SERS spectra for (A) 100 ng/mL cocaine in gold-doped sol-gel capillary and (B) 50 µg/mL cocaine in silver-doped sol-gel capillary to (C) Raman spectrum of pure cocaine, using laser at 785 nm and 8 cm<sup>-1</sup> resolution. Conditions: (A and B) 75 mW power laser, 250 µm diameter spot, 1 min acquisition and (C) 300 mW power laser, 5 min acquisition. With permission from [24].

To demonstrate the field measurements capability, cocaine was also measured with a hand-held dispersive Raman analyzer, operating with the same laser excitation. At first, samples of cocaine diluted in water were measured to determine the sensitivity of the analysis in a non-interfering matrix. Later, oral fluid samples were collected using oral swabs, spiked with a small amount of aqueous drug and measured within one hour from the collection. As expected, the LOD of the drug diluted in water was better (50 ng/mL) than the one in the oral fluid (10 µg/mL). In fact, oral fluid contains glycosylated salivary mucins that can trap drug molecules, because of their viscous nature, and clog the sol-gel pores blocking the metal surface. To break-up the mucins, acetic acid was added to samples. The cocaine was separated from the oral fluid by solid-phase extraction (SPE), placing functionalized silica particles in a capillary column used in-line with the SERS-active capillary. After the SPE treatment of doped oral fluid samples, lower LODs were measured (see table 1) and, furthermore, the height of the most intense cocaine peak (999 cm<sup>-1</sup>) resulted approximately two times higher than the one in the water. It suggests that SPE not only separates the drug from the oral fluid but also concentrates it, permitting to detect cocaine in oral fluid at a concentration of 50 ng/mL. To test these results, SPE treatment was also applied to cocaine samples in water and resulted that, in this case, the height of the most intense peak was about four times higher than the one without the pretreatment. This data indicated that not all of the cocaine was successfully extracted from the sample [24].

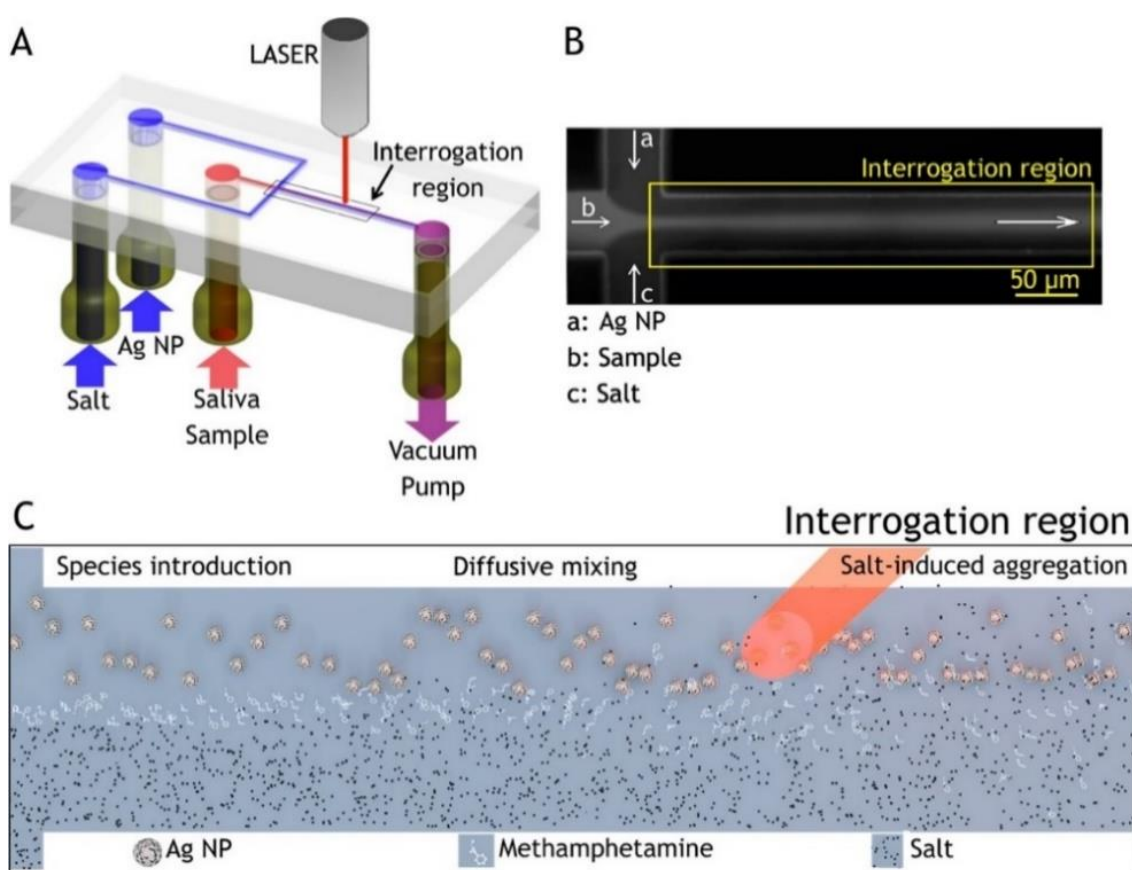
Thanks to its usefulness, this approach was utilized by the same research team, in order to develop a portable device capable to correctly identify overdose drugs in patients in emergency room or in ambulance prior to their arrival at the hospital [25]. For this reason, the device had to be easy to use, non-invasive and rapid, allowing to realize analysis in just few minutes. First, a searchable SERS spectra library was prepared by measuring the spectra of about 150 drugs using a conventional Fourier Transform Raman spectrometer with a 785 nm laser excitation. Later, oral fluid samples were collected with a swab, doped with the drug after the addition of

acetic acid to break the oral fluid mucins, and injected in a SPE capillary connected in-line with a gold-doped sol-gel capillary. Then, their spectra were measured by a hand-held Raman analyzer operating with the same laser excitation of the conventional spectrometer (785 nm), to demonstrate its capability to be used for emergency room measurements. This study showed that, creating previously a spectra library, it was possible to identify a drug simply comparing its entire spectrum to those of the library (without the necessity of identifying the peaks one by one). Furthermore, the identification process could be automatized by using a mathematical treatment. For this purpose, four algorithms were compared: Absolute Value, Least Squares, Euclidean Distance, and Correlation (that resulted the best). The first two algorithms subtracted the measured spectrum from each library spectrum, while the second two algorithms divided the measured spectrum by each library spectrum and subtracted the results from 1 to conform to the first two algorithms. Then, the results were reported as a Hit Quality Index (HQI) where a perfect match would result in  $HQI = 0$  and a complete mismatch (no peaks in common) would result in  $HQI = 1$ . The method was optimized for cocaine, leading to detect a concentration of 50 ng/mL (see table 1), but also other typical overdose drugs like phencyclidine, diazepam and acetaminophen were measured detecting values between 1 and 10  $\mu\text{g/mL}$ . The entire analysis, from the sample collection to the positive or negative identification was performed in less than 10 minutes, demonstrating the applicability of the portable device for oral fluid analysis in ambulance or in emergency room [25].

Lately, the same research group developed a sampling kit composed of an oral fluid collector, a SPE capillary and a SERS capillary used with a portable Raman spectrometer. The final aim of this work, focused on the detection of diazepam in oral fluid, was to be able to perform roadside drug tests and identify impaired drivers [26]. Although the diazepam is a therapeutic drug, it is often consumed in illicit quantities affecting motor functions, such as coordination and balance and so adversely affects driving. The usefulness of this device in roadside tests was underlined considering that diazepam is a fast acting drug, which means that major plasma levels occurred between 30 and 90 minutes after oral administration, so in situ analysis may be critical to establish if and when the drug was ingested. As in the previous studies, a 785 nm diode laser was used and SERS effect was provided by silver and/or gold nanoparticles incorporated in a porous glass structure. The first measurements were made to determine which of the two metals provided the best enhancement effect. The gold was shown to produce a better spectrum in comparison with the one obtained with the silver, so all the subsequent analyses were made with gold-doped sol-gel capillaries. Measuring different diazepam concentrations in water, it was found that the lowest concentration of drug detectable was 125 ng/mL. Subsequently, the same analyses were conducted spiking oral fluid samples with diazepam. The SERS effect was further enhanced by adding acetic acid to the sample, to chemically separate the drug from the saliva mucins, and passing the sample through a 0.2 micron filter, to physically separate the unbound drug from the mucins. By this approach, the least concentration of diazepam detected in oral fluid was of 50  $\mu\text{g/mL}$ . To lower the LOD, they modified the sample pre-treatment evaporating the solvent from the eluate, in order to concentrate the sample prior to add it into the SERS-active capillary. All the analysis required about 15 minutes and permitted to detect concentrations of diazepam until 100 ng/mL (see table 1) in spiked oral fluid [26].

Recently, another article has been published focused on the development of a SERS-microfluidic device (chip) capable to detect trace concentrations of illicit drugs in oral fluid samples [27]. In this case, to demonstrate the efficiency of the method described, the principal purpose was to detect methamphetamine, despite its low affinity with the silver. The choice is probably due to the growing use of this drug at particularly rapid rates, because of its low cost and ease of

manufacture. The developed chip controlled and optimized the interaction of microliters of oral fluid samples with a SERS substrate based on silver nanoparticles (Ag-NPs) in suspension, in presence of lithium chloride as aggregation-inducing salt. In the design of the device three streams were present, containing respectively the analyte, the salt and the nanoparticles. The spatial arrangement and the flow rate of the various streams were customized for optimal SERS detection. Figure 2 shows a scheme of the device (A), the flow- focusing junction (B) and the characteristic diffusion profile of the sample (C). The oral fluid sample, the salt solution and the Ag-NPs suspension are introduced in the device and driven through in by a vacuum pump. Then, at the junction, the drug molecules in the central stream start to diffuse laterally into the side streams. Authors explained that the separation of the analyte from the complex fluid was possible because any larger species that may be present in the sample will diffuse more slowly. The Ag-NPs were much bulkier than any of the other chemical species involved in the process and they diffuse much more slowly than the molecular species.



**Figure 2.** Flow-focusing microfluidic device used for controlled silver nanoparticles (Ag-NP) aggregation. (A) Introduction in the device of Ag-NP suspension, oral fluid sample and salt solution which are driven through it by a vacuum pump. (B) Flow-focusing junction. (C) Schematic of the reaction. With permission from [27].

Nevertheless, different sample concentrations could cause aggregation at different rate, so they decided to perform an aggregation which should create a reproducible reaction independent of the analyte concentration. The salt ions, which play the role of aggregating agent, diffused quickly, but they had to travel a greater distance before reaching the other side stream (where the Ag-NPs resided) because they crossed the central stream. Thus, significant Ag NP aggregation only took place after the analyte had sufficient time to adsorb on the nanoparticles.

However, the use of an aggregating agent presented the disadvantage that Ag-NPs accumulated in the channel and adhered on its walls. Therefore, the given device had to be used maximum for about 10 minutes and only the data collected within that time had to be considered.

In addition, a numerical simulation of the system was made after SERS measurements. It was shown that the strongest SERS signal was observed at a location downstream where abundant aggregates were present. They even observed that the SERS signal also depended considerably on the state of aggregation of the nanoparticles (dimers, trimers, etc.). Collected data were also statistically treated creating a chemometric model of Principal Components (PCs) and corresponding Latent Variables (LVs), which showed to be a good tool for the automated classification of the spectra. In spite of the limitations previously reported, authors stated that the cartridge-based microfluidic system developed was capable of performing a reproducible detection of methamphetamine in an oral fluid sample with a LOD of 1.5 ng/mL (see table 1) in just few minutes, which makes it potentially useful for forensic applications [27].

The latest study published, aimed to the detection of illicit drugs in oral fluid by SERS, was performed employing gold nanoparticles (Au NPs) dotted magnetic nanocomposites (AMN) modified with inositol hexakisphosphate (IP6) as SERS substrate [28]. Actually, in this case, the goal was to detect drug-related biomarkers in oral fluid and fingerprints to be able to indicate the amount of the parent drug present in the original sample. The two substances investigated were cotinine as metabolite of nicotine and benzoylecgonine as metabolite of cocaine. The dislocations or sharp discontinuities on a metal surface, or the junction of two or more metal nanoparticles, from which SERS enhancement arises, are regarded as “hot spots”. In this case, the utilization of a magnet to reversibly control AMN for generating favorable density of hot spots via the optimization of aggregation was studied. Briefly, AMN were prepared synthesizing Fe<sub>3</sub>O<sub>4</sub>-NPs via the chemical coprecipitation method aided by IP6 and then dotted with Au-NPs by the chemical reduction of 1% chloroauric acid with 1% sodium citrate. In the structure of the resultant AMN, the IP6 acts as a bridge between Fe<sub>3</sub>O<sub>4</sub>-NPs and Au-NPs, and the total amount of Au was about 40.6%. Some trials with probe molecules were carried out in a capillary, which demonstrated that when a magnetic field was applied in situ on the AMN with the molecules, the SERS signal underwent a great enhancement phenomenon. By placing a magnet directly under the capillary, the aggregation happened within 4 minutes because AMN were composed of many Fe<sub>3</sub>O<sub>4</sub>-NPs and Au-NPs as a whole system. Subsequently, the aggregate was redispersed by simple sonication and the Raman signal returned to the level of original intensity, showing that the applied process was reversible and so easily controllable. By using a portable Raman spectrometer with an excitation wavelength of 785 nm and a laser power of 300 mW, the same measurements were performed on aqueous solutions of cotinine, nicotine and benzoylecgonine showing a LOD of 0.88 ng/mL, 0.81 ng/mL and 14.5 ng/mL respectively. Finally, doped oral fluid samples were analyzed after adding 0.5 mL of pure water for facilitating the magnet inducing and, as expected, higher LODs were detected (see table 1). For the cotinine the LOD was 8.8 ng/mL and for the benzoylecgonine was 29 ng/mL. Also in fingerprints the LOD of cotinine achieved was lower than the one previously reported. Then, it could be concluded that a portable Raman analyzer AMN-based and a magnetically optimized technique could be employed as in situ protocol to screen drugs of abuse traces in biological fluids and to differentiate the drug addicts from the smokers [28].

## ***IR spectroscopy***

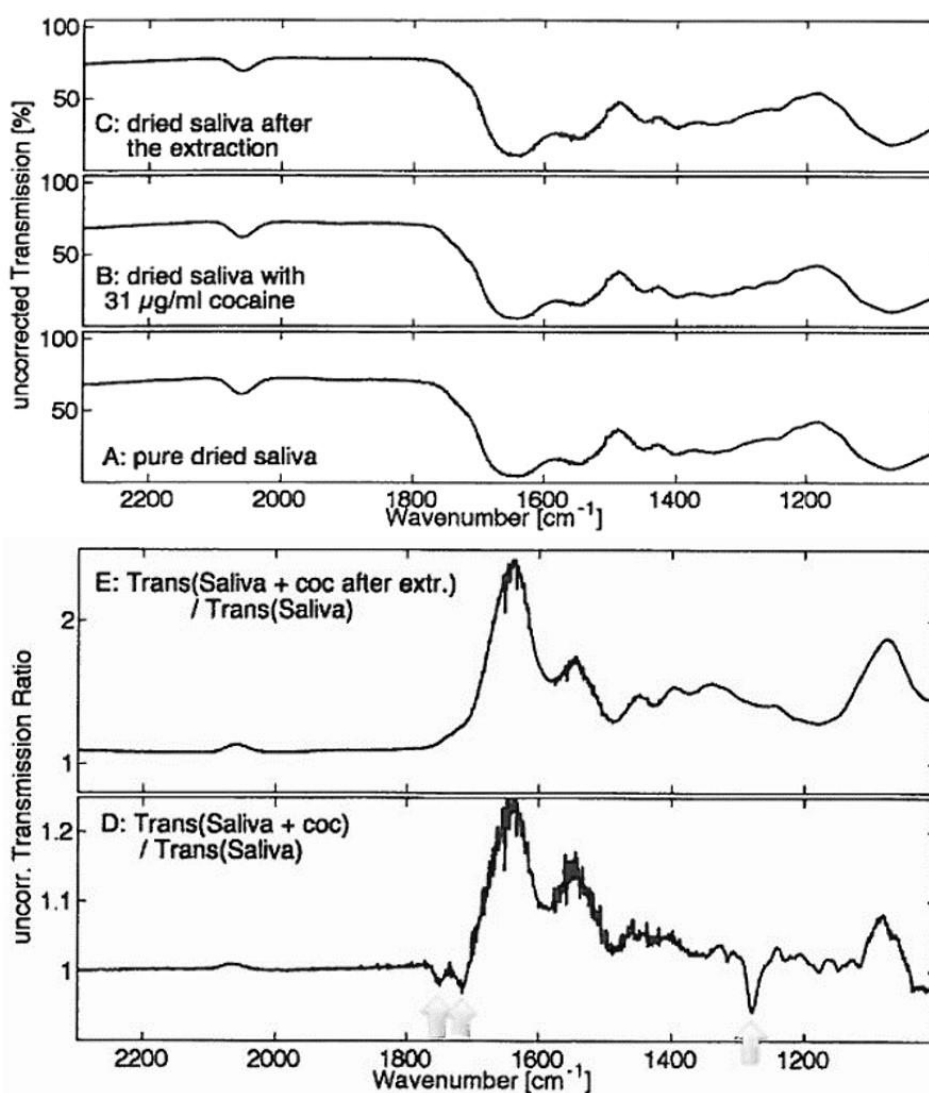
IR spectroscopy is an analytical technique that explores the IR region of the electromagnetic spectrum, comprised between 14000  $\text{cm}^{-1}$  and 10  $\text{cm}^{-1}$ , which is divided in near-, mid- and far-infrared in relation to the visible spectrum. The IR signal is a consequence of the absorbance of the light due to molecular vibrations, so also overtones and combination of vibrations could be observed in the spectrum of a sample. The IR spectroscopy is a very fast technique, in fact, with the modern instruments like Fourier transform infrared (FTIR) spectrometers, a spectrum could be easily recorded in few seconds. Furthermore the technique is not destructive because a sample can be analyzed without or with a minimal pretreatment allowing to avoid many analytical steps (that could be sources of errors), and to reuse the sample after the measurement. Moreover, with this technique, it is also possible analyze packaged samples without removing the packing. The main difficulty of this technique is the complexity of the spectra: in complex matrices it is possible that various absorption bands could overlap, making uneasy to identify different substances possibly contained in the sample [29].

The use of IR spectroscopy to determine drugs in oral fluid is rapidly growing in recent years so, as in the previous case, few papers have been published up to date (see table 2).

In a first study, Hans et al. [30] presented IR spectra of cocaine in water and human oral fluid exploring, in addition, the possible spectral interferences of diluents, masking substances or common medicine. They evaluated the most appropriate absorbance range with the final goal to develop a compact and easy-to-use device for direct semi-quantitative drug testing. Attenuated total reflection-infrared (ATR-IR) spectroscopy coupled with microfluidics was employed. They worked in the mid-IR region and collected all the spectra with a commercial FTIR spectrometer (operating between 2300 and 900  $\text{cm}^{-1}$ ) and with an ATR unit. The most appropriate spectral range for strong cocaine absorption was found to be between 1800 and 1710  $\text{cm}^{-1}$ . All the other substances analysed, instead, had a strong absorption in other ranges of the spectrum, which means that they interfered minimally. For aqueous solutions a spectra treatment was necessary to subtract the great signal of the water in the mid-IR region. In order to verify the possibility to detect drugs in oral fluid, an oral fluid sample from a fasting person was collected as representative of the simplest matrix spectrum. Characteristic absorptions were observed in 1100-1000  $\text{cm}^{-1}$  and 1650-1550  $\text{cm}^{-1}$  regions, which were attributed, respectively, to sugars and proteins. In fact, analyzing the same samples after storage in fridge for several days, the major changes were observed in those ranges, due to proteins degeneration and sugars reduction. By measuring the spectrum of a cocaine-doped sample of oral fluid, it was reported that the LOD was very high ( $> 400 \mu\text{g/mL}$ ) because of the strong absorption of the water. This problem was partly resolved by drying the sample and a LOD of 20  $\mu\text{g/mL}$  (see table 2) was obtained. However, since the drying pattern of the oral fluid on the ATR unit differed significantly, there was a less accuracy in the results and only qualitative (or semi-quantitative) measurements were allowed. Finally, considering that in real life not always persons have empty stomach, also the influence of mouthwashes, alcohol and other interfering substances was studied. The new spectra obtained showed some differences, but the studied substances did not impede the detection of cocaine. Additionally, alcohol concentration and its temporal evolution were easily detected in oral fluid [30].

A subsequent work was aimed to develop a simple one-step extraction protocol to extract cocaine from oral fluid in an almost IR transparent solvent, and to successfully record its spectra. A commercially available FTIR spectrometer with an ATR crystal, operating between 2300 and 900  $\text{cm}^{-1}$ , was used [31]. Initially, a cocaine-doped oral fluid sample was spread on the crystal

surface of the ATR unit and two spectra were recorded, before and after the sample dried up. Clearly the second spectrum resulted better than the first one. In this case, in fact, the analyte was in contact with the surface, closer to the origin of the ATR source and the evaporation of the water had eliminated its interfering absorption. Again, even if a LOD of 20  $\mu\text{g/mL}$  was reached, the different drying pattern of the samples changed the obtained results, making this method only semi quantitative. The extraction of cocaine from oral fluid, to improve the LOD obtained, was then made using perchloroethylene (PCE). Figure 3 shows the change between the spectra of the dried oral fluid before (A) and after (B) the spiking with cocaine and after the extraction of the cocaine (C). It could be also observed that the characteristic peaks of cocaine present at 1280  $\text{cm}^{-1}$  and between 1760 and 1710  $\text{cm}^{-1}$  (marked with grey arrows) are visible only in spectra obtained from the ratio between the spectrum of the oral fluid spiked with cocaine and the one of simple oral fluid (D). Instead, no peaks were observed in the spectrum obtained by the ratio between the spectrum of the spiked oral fluid sample after the extraction of cocaine and the one of simple oral fluid (E).



**Figure 3.** Spectra of oral fluid (saliva) of one test person. The three major cocaine absorption peaks are marked with grey arrows. Conditions: FTIR-ATR spectrometer with Quantum Cascade Laser, resolution 1  $\text{cm}^{-1}$ . With permission from [31].

The vanishing of the cocaine specific peaks demonstrated a successful extraction and in fact, collecting the spectrum of dried PCE phase after the extraction, cocaine peaks were clearly visible. If the cocaine was extracted from oral fluid right after the collection and preparation of the sample, a good reproducibility could be observed. It was concluded that this method not only allowed to reach a LOD of 10  $\mu\text{g/mL}$  or lower after extraction in PCE (see table 2), but also allowed to overcome the problem of concentration changes due to the small variations in the dried sample pattern [31].

In a successive study [32], the same research team focused on the preparation and set up of reference samples, and analyzed them with IR laser spectroscopy employing two different approaches. Basing on the idea that an extraction process was necessary to extract cocaine from the oral fluid to an IR-transparent liquid (PCE), prior to its IR detection, the reference samples were prepared dissolving cocaine in PCE. The first approach utilized an ATR unit and analyzed dried samples, while the second utilized a transmission cell and analyzed liquid samples. In both approaches, a quantum cascade laser (QCL) was employed as light source. QCLs are used with a narrow emission bandwidth at a wavelength corresponding to an absorption line of the molecule of interest. In this case the QCL was set at a wavelength of about 1750  $\text{cm}^{-1}$  (corresponding to a high cocaine absorption) in order to differentiate the drug from other minor substances present in oral fluid. The spectra of other substances, in fact, could interfere with the one of the drug in analysis leading to a confusion among peaks. The comparison between the results obtained with the two techniques showed that the cocaine LODs in oral fluid were different. The limit achieved with ATR corresponded to 3  $\mu\text{g/mL}$  and was lower than the one reached with the transmission cell corresponding to about 10  $\mu\text{g/mL}$  (see table 2). However, in the first case the variability of the results was higher because of the use of dried pattern-variable samples [32].

Subsequently, to perform a portable microsystem for detecting cocaine in oral fluid, Wägli et al. [33] developed a solution for microfluidic sample pretreatment to elute cocaine from the oral fluid to the PCE, by using a droplet-based liquid-liquid extraction process. In this work, the authors described in detail the fabrication process of the optical waveguide system in a chip [34] and the complex extraction process that finally led to the optical detection of the drug. The lowest concentration of cocaine, detected in oral fluid after extraction in PCE on the microfluidic system developed, was even 500  $\mu\text{g/mL}$  (see table 2). However, this work demonstrated that, despite the very high LOD, a quickly quantification of cocaine in oral fluid was possible by using an opportunely structured microdevice [33].

Another study about the analysis of cocaine and street cocaine in oral fluid was made by Hans et al. [35] and showed another important step towards the development of a compact, easy-to-use, portable, quick and semi-quantitative screening device based on IR detection. In their previous work [30] they introduced the FTIR-ATR spectroscopy without sample preparation to study spectral interferences and to explore the LOD of cocaine in oral fluid. A high influence of the IR absorbance of water throughout the region of interest for cocaine detection, in particular between 1800 and 1200  $\text{cm}^{-1}$ , had been observed. In this work, they pursued to demonstrate that it is possible to decrease the LOD previously obtained (20  $\mu\text{g/mL}$ ) reducing the background by a simple one-step extraction performed mixing the spiked oral fluid with an extraction solvent on a microfluidic system. All spectra were acquired with a FTIR spectrometer combined with an ATR unit and displayed in the range between 1000 and 1850  $\text{cm}^{-1}$ . The results showed that it was difficult to detect small quantities of cocaine in dried samples but the problem could be overcome by applying an extraction process. As in the previous papers, PCE was chosen as

extraction solvent because it was poorly soluble in water and had a small absorbance in the cocaine IR absorption range. Clearly, the spectra of the extracts in PCE showed a higher signal to noise ratio, so cocaine was detected in oral fluid without comparing the spectrum with that of an unspiked sample. Furthermore, no interferences with the analyte by any extractable endogenous component of the oral fluid were observed. Not even major differences were observed between female, male, smoker or non-smoker oral fluid samples. After the extraction process, cocaine LOD found was of 1.3  $\mu\text{g/mL}$  (about 20 times the value without extraction) because cocaine dissolves much better in PCE than in oral fluid. They also demonstrated that it was possible to pre-concentrate cocaine during the extraction process, leading to detect cocaine at concentrations of few hundreds  $\text{ng/mL}$  (see table 2). Finally, all the analyses were repeated on samples of oral fluid spiked with street cocaine and it was observed that some of the adulterant of street samples were visible in the IR absorbance spectrum. According to these observations, authors stated that the developed device was simple to use, provided reproducible results in a short time (similar to that of the currently used immunoassays) and do not need of a bulk equipment. Thanks to these characteristics the device could be of great utility in roadside tests to obtain (semi-)quantitative results allowing the evaluation of the risk for impaired drivers [35].

Recently, another paper was published by Jouy et al. [36] in which two sensor based on QCL absorption spectroscopy are developed for trace substances detection in liquids and gases. The detection of cocaine in oral fluid was chosen as a meaningful possible demonstration for the liquid sensor. As a consequence, the single mode emission spectra were centered around 1720  $\text{cm}^{-1}$ , because it is a good region for cocaine identification, without interferences from other molecules possibly present in oral fluid. In the first part of the study, the sensor used to target low concentrations of cocaine in oral fluid was the same that the authors had used in their previous work [33]. It was made of four main parts: a QCL as a MIR source, a Si/Ge waveguide on top of which a micro-fluidic system is bonded to extract cocaine from oral fluid to PCE and a commercial detector. In this case, the Si/Ge waveguide was designed to have an important evanescent field penetrating into the fluid flowing above it and was processed in an L-shape so that the detector is not in the same direction as the laser. When cocaine was directly dissolved in PCE, it could be achieved a LOD of 100  $\mu\text{g/mL}$ , while when the cocaine was dissolved in oral fluid and then extracted, the LOD observed was 500  $\mu\text{g/mL}$ . Anyway, by interpolation of the signal-to-noise ratio, it could be reached an estimated LOD of 100  $\mu\text{g/mL}$  (see table 2), indicating a successful liquid-liquid extraction in the microfluidic system. In the second part of the study, to improve the LOD of the sensor, an S-shape waveguide with two 180° bends has been performed. Increasing the contact surface between the waveguide and the solvent containing the drug, it was possible to reach a LOD lower than 10  $\mu\text{g/mL}$  when cocaine is directly dissolved in PCE. By integrating this and others improvements, such as split the waveguide or pre-concentrate the cocaine in the extracting solvent, the authors expected to reach lower LODs of cocaine also in oral fluid [36].

The last reported work about the analysis of illicit drugs in oral fluid [37] was focused on the detection of traces of ecstasy (MDMA) by using two different techniques. At first, ion mobility spectrometry (IMS) after thermal desorption was used. Later, IMS and IR spectroscopy after liquid-liquid microextraction (LLME) were employed jointly to enable the detection and double confirmation of MDMA abuse. IMS spectrometry is based on the gas-phase separation of ionized analytes under a weak electric field at ambient pressure by their mobility in the drift gas. The analytical potential of IMS derives from its high sensitivity and operational speed. The joint use of two analytical techniques based on different chemical principles such as IMS and IR



spectroscopy affords accurate and fast detection, identification and semi-quantitation of abuse drugs. In this work, the direct analysis of oral fluid samples by IMS after thermal desorption, required the addition of acetic or hydrochloric acid to the samples to facilitate the desorption and the ionization of the analyte. MDMA concentrations were calculated by interpolation into a calibration curve constructed from solutions spanning the range 200-1600 ng/mL and the estimated LOD was 160 ng/mL (see table 2). LLME was performed adding sodium hydroxide and chloroform to the oral fluid samples and vortexing the mixture. After the separation of the two phases by centrifugation, the organic phase was analyzed by IMS. As before, the concentration of MDMA was calculated by interpolating the IMS signal into a calibration curve spanning the range 75 400 ng/mL. The curve was constructed by extracting the analyte from aqueous standards and a LOD of 11 ng/mL was estimated (see table 2). The samples were also analyzed by IR spectroscopy to confirm the identification of MDMA and the spectra were recorded in the ATR sampling mode operating in the mid-IR region (4000-650  $\text{cm}^{-1}$ ). Once again, the MDMA concentrations in positive samples were calculated by interpolation of their signals into a calibration curve spanning the range 0.8 200  $\mu\text{g/mL}$  and the estimated LOD was 320 ng/mL (see table 2). Selectivity, sensitivity and specificity of the applied methodology were so assessed, showing that the joint use of IMS and IR spectroscopy afforded the unambiguous identification of MDMA abuse in oral fluid sample. The protocol, in fact, combined the increased sensitivity of IMS to identify positive samples with the high selectivity of IR spectroscopy to confirm the positive results within an acceptable time (5 minutes) permitting to perform in situ analysis [37].

However, as seen until now, the physical conditions of the sample, the surrounding environment and the overlapping and combination of bands influence the spectra, making difficult the interpretation of the obtained data. For this reason, IR spectroscopy almost always requires to be combined with chemometric techniques that are very useful tools for results interpretation. As a consequence, also in this case, further improvements are necessary to automatize the phase of data processing and thus make the developed devices practically applicable.

### ***NMR spectroscopy***

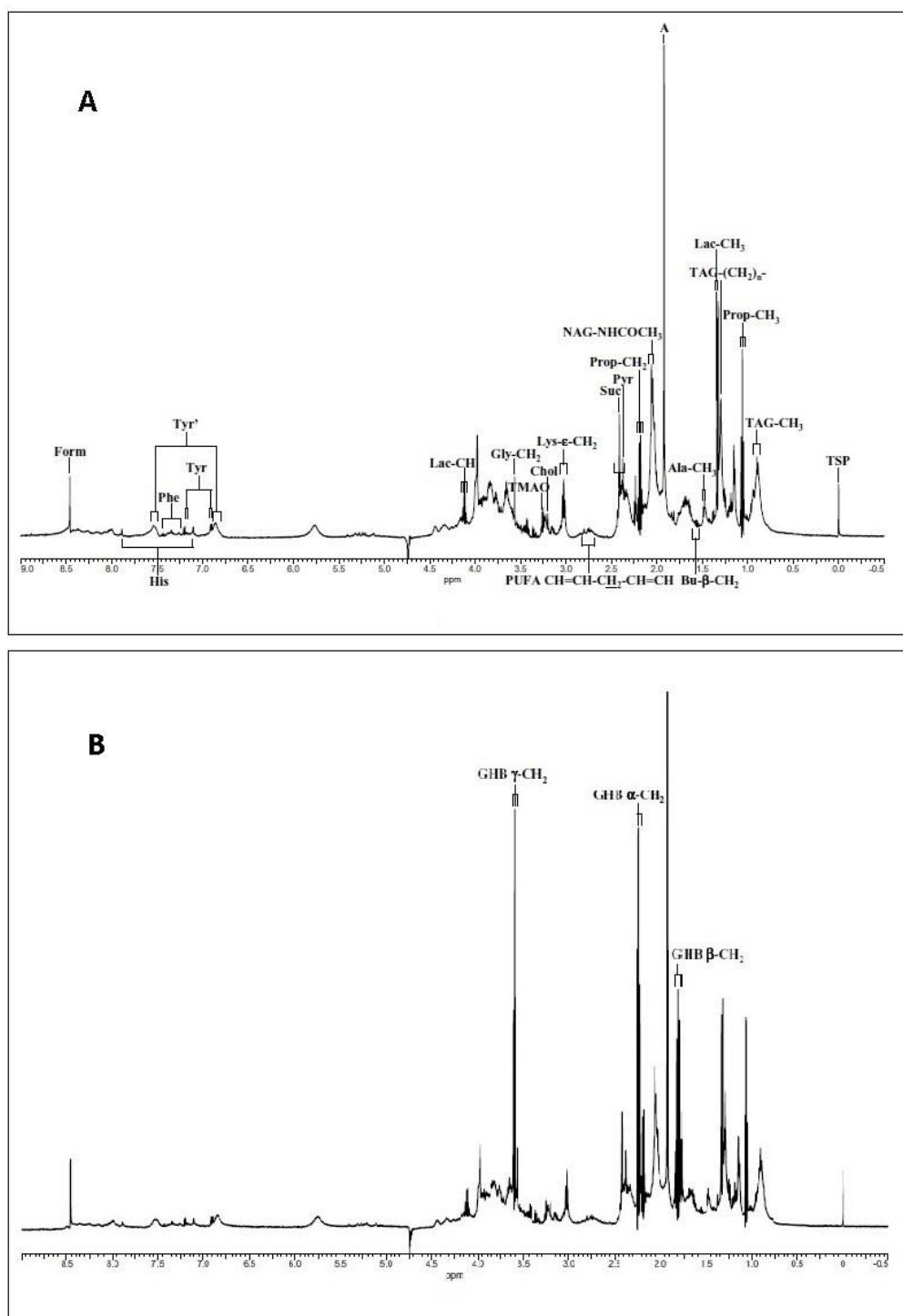
NMR spectroscopy is an analytical technique that gives information about the structure and the chemical environment of the molecules based on the magnetic properties of some nuclei. Only nuclei of atoms and isotopes with odd atomic number and/or odd atomic mass are observable with an NMR spectrometer, because only nuclei that have spin quantum number equal to  $\frac{1}{2}$  show an NMR signal. When a molecule is subjected to a magnetic field (polarization field or  $B_0$ ), the vector obtained from vector sum of the magnetic moments of each nucleus (magnetization vector or  $M$ ) aligns with this field reaching an equilibrium position. If another electromagnetic field (excitation field or  $B_1$ ) with an appropriate frequency is applied, the equilibrium position is lost and the nuclei start to absorb energy, which is re-emitted when the application of  $B_1$  is interrupted. A coil that feels the variations of  $M$  is placed around the sample and connected to a detector, which provides the NMR spectrum collecting the signal until  $M$  reaches again the equilibrium position [38].

This method allows simultaneous analysis and identification of a variety of molecules without the requirement of pretreatments such as extraction, separation or purification, and needs only a minimal preparation of the sample. The technique is highly sensitive, not invasive and the analysis is fast (10-15 minutes).

However, in spite of this, few and old studies about its use for the analysis of oral fluid have been published up to date [39-41]. This is probably due to the difficulty in observing  $^1\text{H}$  signals of substances other than water, because such signals are hidden by the large  $^1\text{H}$  signal of  $\text{H}_2\text{O}$  present in biological samples. In some cases, to obtain a clear spectrum, the broad overlapping resonances due to the presence in biofluids of water and other macromolecules, are suppressed by the application of pre-selected pulse sequences. For example, some differences were found between the  $^1\text{H}$ -NMR spectra of oral fluid specimens from diseased and healthy persons, and before and after intake of alcohol [39].

Until now, only two articles that specifically deal on the determination of illicit drugs in oral fluid and/or in common beverages have been reported. Both of them, are focused on the investigation of the gamma-hydroxybutyrate (GHB) and the corresponding lactone gamma-butyrolactone (GBL). GHB is a very important compound in forensic field because it is used especially at "rave" parties as "sexual assault" or "date rape" drug. This substance is a central nervous system depressant and it is recognized as drug of abuse, commonly available as "Liquid ecstasy" or "Liquid X", since gives rise to euphoria or hallucinogenic episodes. GBL is a synthetic precursor of the GHB (obtained from it thanks to a rapid and reversible conversion) and it was used for the clandestine manufacture of GHB. [42, 43]

Due to the forensic interest of GHB, Grootveld et al. [42] used  $^1\text{H}$  NMR spectroscopy to detect and quantify this compound in oral fluid and in a common beer. At first, the characteristic peaks of the GHB were identified by analyzing an aqueous solution of the analyte at pH 7. It was observed that the three prominent resonances of the GHB were assignable to the  $\alpha$ -CH<sub>2</sub> (triplet,  $\delta = 2.25$  ppm),  $\beta$ -CH<sub>2</sub> (triplet of triplets,  $\delta = 1.81$  ppm) and  $\gamma$ -CH<sub>2</sub> (triplet,  $\delta = 3.61$  ppm) groups. Subsequently, they analyzed oral fluid samples spiked with the drug and found that the intensities of the GHB peaks increased with increasing the added concentration. Figure 4 shows the spectra of an oral fluid sample before and after the addition of 1.3 mg/mL of GHB. It should be noted that the first spectrum contains a wide range of sharp, prominent signals assignable to many low-molecular-mass biomolecules, together with selected exogenous agent. In the second, instead GHB peaks are clearly visible with little or no overlap. Consequently, data acquired in this study demonstrated the ready applicability of this technique to the detection of illicit drugs in complex and multicomponent samples such as oral fluid or beverages. Moreover, it is possible to obtain much information about the molecular nature of both endogenous and exogenous species present in the sample. GBL was not directly investigated but the spectra collected demonstrated that the precursor is readily detectable and easily distinguishable from GHB by observing the chemical shifts [42].



**Figure 4.**  $^1\text{H}$ -NMR spectra of an oral fluid sample (pH value 6.87) (A) prior and (B) subsequent to the addition of 1.3 mg/mL of GHB. Conditions: 600.13 MHz single-pulse. With permission from [42].

To resolve the problem of the interfering NMR signal of water, Lesar et al. [43] studied another approach to suppress water bands, although in this case GHB and GBL were studied only in common alcoholic beverages. They used the innovative Presaturation Utilizing Relaxation Gradients and Echoes (PURGE) method coupled with  $^1\text{H}$  NMR spectroscopy. This method has the advantage that is easy to carry out even for non-spectroscopists, but presents the disadvantage that labile protons are suppressed in the spectrum. NMR spectra were recorded at different concentrations and the collection times were varied to obtain a signal-to-noise ratio

above 10. This was used to determine the sensitivity of the method and to optimize the time spent using the spectrometer. Quantification was done plotting the ratio [integral of the drug multiplet/integral of the internal standard singlet] versus drug concentration. The PURGE method was used to verify if the technique permitted to determine the quantity of GHB formed by the conversion of GBL. Data obtained demonstrated that the technique was capable to determine the relative concentration of both drugs only when the samples were spiked with GBL. In fact, the conversion from GBL to GHB happened at different rates, depending on the pH, until the equilibrium was reached. Instead, when the beverages were spiked with the sodium salt of GHB, the conversion from the basic to the acidic form through the exchange of a proton was so rapid that NMR was not able to distinguish the two forms. Authors also stated that, if quite large concentrations of drugs are present, it is possible to easily determine the origin of the GHB present in a seized sample without changing the matrix. In fact, the provenience of GHB from GBL or from the sodium salt could be simply determined from the shift of the signal in the same sample of beverage [43].

As all these studies were conducted using individually  $^1\text{H}$ - or  $^{13}\text{C}$  NMR spectroscopy, authors also proposed to improve results by using bidimensional NMR techniques such as Total Correlated Spectroscopy (TOCSY), J-Resolved Spectroscopy (JRES) or Heteronuclear Multiple Quantum Coherence transfer spectroscopy (HMQC). In fact, it has been already reported that the use of a combination of 1D and 2D NMR spectroscopy permitted a better simultaneous examination of a very wide range of components present in oral fluid [40]. Hence, the employment of bidimensional NMR appears a promising tool to improve the technique and then to be able to determine the presence of illicit drugs in oral fluid.

## **Conclusions and future trends**

Spectroscopic methods appear to be an excellent combination of fast and non-destructive tools, although until now they have been scarcely used for drugs detection in oral fluid. Actually, they still need improvements to be applied for the determination of a wide range of drugs in real samples and to be of real interest for clinical and forensic applications. Furthermore, all the spectroscopic approaches developed up to date and discussed in this review pursue more qualitative than quantitative measurement. In fact, analytical parameters such as selectivity, specificity, precision, linear range, robustness, accuracy, matrix effect and reproducibility have not been closely studied. However, the apparent growing interest in these techniques will make them useful in the near future in different fields.

In particular, up to date, Raman spectroscopy in SERS working mode has been shown as the most sensitive spectroscopic technique for the detection of illicit drugs in oral fluid permitting to reach LODs of 50 ng/mL for cocaine, 29 ng/mL for benzoylecgonine, 8.8 ng/mL for cotinine and even 1.5 ng/mL for methamphetamine. The use of IR spectroscopy for the detection of drugs of abuse in oral fluid is growing, although LODs obtained (few hundreds ng/mL in the best case) do not yet satisfy the necessities in forensic and clinical fields. Nevertheless, it should be highlighted that, by coupling IR spectroscopy with IMS spectrometry after LLME, it has been possible to confirm the presence in oral fluid of MDMA even in a concentration of 11 ng/mL. Finally, NMR spectroscopy has been seldom used to determine drugs in oral fluid and the attention has been mainly focused on the study of the NMR spectral information provided.

This review has therefore shown how vibrational spectroscopic techniques, especially SERS, seem to be promising approaches for quickly achieving low LODs and for providing confirmatory results.

Another future trend seems to be related with the use of portable instrumentation, which would permit to perform in situ analysis with important implications in forensic and clinical fields. A rapid analysis of oral fluid will overcome the problem of the rapid drug metabolism. In fact, since the process causes the variation of drug concentration as the time passes, it can affect the results making them not reliable. Furthermore, those techniques that already dispose of good portable instrumentation, such as Raman and IR Spectroscopy, seem to be the most promising spectroscopic tools for the determination of illicit drugs in oral fluid.

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